Plant Physiology and Biochemistry 133 (2018) 29–39

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Kaolin particle film modulates morphological, physiological and biochemical olive tree responses to drought and rewatering

Cátia Brito, Lia-Tânia Dinis, Helena Ferreira, Luís Rocha, Ivo Pavia, José Moutinho-Pereira, Carlos M. Correia*

CITAB, Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Universidade de Trás-os-Montes e Alto Douro, 5000-801, Vila Real, Portugal

ARTICLE INFO

Keywords:
Drought
Recovery
Photosynthesis
Mitigation strategies
Oxidative stress
Antioxidants

ABSTRACT

Regarding the foreseeing climate change is reasonable to expect harmful consequences to olive tree (*Olea europaea* L.), an iconic species of Mediterranean region. Thus, the selection of practices that allow a better drought resistance and recovery capacity needs the immediate attention of scientific community. This study evaluates the strategies adopted by young potted olive trees, subjected to three cycles of drought and rewatering, in the presence of a reflective clay, kaolin (KL). The results demonstrated that KL induced shade-related leaf structural changes and was effective in keeping leaf water status during the most stressful periods. In general, photosynthetic activity of sprayed plants was improved by the alleviation of drought-induced stomatal and non-stomatal limitations. Moreover, during stress imposition sprayed leaves showed reduced oxidative damages, allowing lower investment in antioxidant defences. Furthermore, sprayed plants also had lower nighttime water losses due to inferior nighttime stomatal conductance, and are able to maintain higher respiration rates. Upon rewatering, the shaded effect conferred by KL limited gas exchange restauration, but improved the plants' capacity to restore the metabolic functions. In spite of the induced physiological and biochemical changes, no significant differences were found in whole-plant water use efficiency and plant biomass accumulation, possibly by the attenuation of photosynthesis restauration during the recovery events. In conclusion, the changes induced by KL might be beneficial under severe conditions, as on realistic Mediterranean field environments.

1. Introduction

In the current settings, olive tree growing under the typical Mediterranean semi-arid conditions are already affected by multiple environmental constraints factors, as drought stress is commonly associated with high temperature and irradiance. Moreover, severe summer conditions and extreme climatic events are predicted to increase in frequency in most future climate scenarios (IPCC, 2013). Although olive is a crop well-adapted to harsh conditions, water deficit has negative repercussions on water relations, carbon assimilation, oxidative pathways, nutrient uptake and biomass accumulation (Bacelar et al., 2006, 2007). In addition, the presence of simultaneous abiotic stresses, as heat and high irradiance levels, can exacerbate drought effects, affecting plant growth and yield and consequently the economic viability of the olive sector. Therefore, is important to increase trees ability to use the scarce available water and the low and unexpected rainfall during the summer.

Undeniably, the recovery of plant functions when water shortage is relieved is crucial to restart growth under drought and rewatering events. Recovery after stress is a very complex process involving the rearrangement of many metabolic pathways to repair drought-induced damages and restore plant growth and productivity (Chen et al., 2016). In olive tree, it has been identified a conservative bearing after rewatering, as trees restoring rapidly the water status along with a slow recovery of stomatal conductance (Perez-Martin et al., 2014). Although have been suggested that drought recovery may play a more important role than drought resistance (escape, avoidance and tolerance) in water deficit response (Chen et al., 2016), the capacity for recovery after successive drought and rewatering cycles have poorly been studied. Those evidences highlight the importance to adopt agronomic practices that allow a better drought adaptability, i.e. the capacity to integrate both drought resistance and recovery capacity (Chen et al., 2016) of rainfed olive orchards. Kaolin, which main constituent is kaolinite, is a white mineral chemically inert (Glenn and Puterka, 2005) that have been proved to be efficient in summer stress alleviation. Once sprayed on the leaf surface, water evaporates leaving a protective particle film

* Corresponding author.
E-mail address: ccorreia@utad.pt (C.M. Correia).

https://doi.org/10.1016/j.plaphy.2018.10.028
Received 27 May 2018; Received in revised form 29 September 2018; Accepted 24 October 2018
Available online 25 October 2018
0981-9428/

Abbreviations

C	control
KL	kaolin
PAR	photosynthetic active radiation
UV	ultraviolet radiation
IR	infrared radiation
DP	drought period
RP	recovery period
RWC	relative water content
LMA	leaf mass area
TS	total section
SP	spongy parenchyma
UPP	upper palisade parenchyma
LPP	lower palisade parenchyma
UE	upper epidermis
LE	Lower epidermis
UC	upper cuticle
TL	trichome layer
A_n	net photosynthetic rate
g_{day}	daytime stomatal conductance
E_{day}	daytime transpiration rate
A_n/g_{day}	intrinsic water use efficiency
C_i/C_a	ratio of intercellular to atmospheric CO_2 concentration
g_{night}	nighttime stomatal conductance
E_{night}	nighttime transpiration rate
$C_{i\text{-}night}$	nighttime concentration of CO_2 in intercellular spaces
R_{night}	nighttime respiration rate
PSII	Photosystem II
F_0	minimal fluorescence
F_v/F_m	maximum quantum efficiency of PSII
qP	photochemical quenching
F'_v/F'_m	capture efficiency of excitation energy by open PSII reaction centers
ΦPSII	effective quantum efficiency of PSII
$E_{cuticular}$	cuticular transpiration
$PE_{w\text{-}night}$	plant whole-nighttime transpiration
$PE_{w\text{-}daytime}$	plant whole-daytime transpiration
$Chl_{(a+b)}$	total chlorophylls
Chl_a/Chl_b	chlorophyll a/chlorophyll b ratio
TAC	total antioxidant capacity
ROS	reactive oxygen species
TLA	total leaf area
BI	total biomass increase
WUE_{WP}	whole-plant water use efficiency
RAI	relative alleviation index
NAR	net assimilation rate
$VPDl_{eaf\text{-}air}$	leaf-to-air vapor pressure deficit
VPD	vapor pressure deficit
PPFD	photosynthetic photon flux density

that increases the reflection of excess radiation (photosynthetically active, PAR, ultraviolet, UV and infrared, IR), avoiding the accumulation of heat load and reducing the risk of leaf and fruit damage from high temperatures and solar injury (Glenn and Puterka, 2005). In general, positive effects were recorded in plant water status, photosynthetic responses and yield, but the results appear to be largely influenced by a set of factors, such as species and cultivars, environmental conditions, plant age and structure (Denaxa et al., 2012; Glenn, 2009; Jifon and Syvertsen, 2003; Nanos, 2015; Rosati et al., 2006; Roussos et al., 2010).

Hence, with this study we aimed to evaluate if kaolin application alleviates the negative effects associated to cyclic water deficit events. For this propose, we studied the effects of kaolin application on (i) physiological and biochemical variables under drought and rewatering; (ii) on leaf anatomical traits; and (iii) on growth responses.

2. Materials and methods

2.1. Plant material and experimental set-up

The study was carried out in Vila Real, Northeast Portugal (41°17′17.83″N, 7°44′12.81″W, 448 m a.s.l.) with own-rooted 3-years-old olive trees (*Olea europaea* cv. Cobrançosa). Plants were grown outdoors in 16 L pots containing a mix of sandy-loam soil and horticultural substrate Siro Oliva (Siro-Leal & Soares SA, Mira, Portugal) (2:1). The surfaces of containers were covered with a thin layer of perlite and sealed with plastic film and aluminum foil. This measure aimed to avoid the evaporation from soil surface and the rain water entering to the pots, and to minimize the temperature increase inside the containers. Pots were randomly arranged and periodically rotated to the neighboring position to minimize the effects of environmental heterogeneity. The climate of the study site is typically Mediterranean-like, a warm-temperate climate with dry and hot summers, classified as Csb according to Köppen-Geiger's classification. Mean annual rainfall is 1023 mm, most of which falls in the autumn-winter with negligible rainfall during the summer months, although 2014 was an atypical summer with some rainfall events (13.7, 11.8 and 13.0 mm during the 1st, 2nd and 3rd recovery periods, respectively. The warmest months are July/August and the coldest months are December/January, with mean daily temperatures of 21.3/21.7 °C and 6.8/6.3 °C, respectively. The maximum, minimum and average air temperature recorded during the experimental period are shown in supplemental Figure 1 (IPMA, 2017).

Prior to the experiment, forty uniform plants, selected based on height, leaf number and leaf area were left for 30 days in the study site for acclimatization, being watered every other day to field capacity, determined gravimetrically. Then, at the beginning of the experiment, 6th July, eight plants randomly chosen were harvested to assess the initial biomass of the different plant organs. The remaining thirty-two plants were subjected to three "drought-rewatering cycles" by withholding water until the occurrence of precipitation (1st and 2nd cycles), or until the stomatal conductance for water vapour (g_{day}) during mid-morning (peak of photosynthetic activity) dropped around 50 mmol m^{-2} s^{-1} (reached at 3rd cycle), a threshold value indicating a situation of severe drought stress experienced by the plants, a value where photosynthetic activity becomes predominantly inhibited by metabolic processes, besides stomatal limitations (Flexas and Medrano, 2002). When occurred precipitation, or when olive trees reached the desired drought intensity, they were re-watered to field capacity, determined gravimetrically, in the evening and also during the following days until A_n was almost restored to well-watered every day control values, as described in a parallel study (Brito et al., 2018). The 1st, 2nd and 3rd "drought-re-watering cycles" had the duration of 12-6 days, 9-3 days and 21-16 days, respectively. Consecutive drought-rewatering cycles simulate what usually occurs under Mediterranean-type ecosystems (Munné-Bosch and Peñuelas, 2003).

Plants were divided in two groups, the first group (C, control plants) was sprayed with distilled water and the second group (KL) was sprayed with an aqueous solution of kaolin (Surround® WP, Engelhard Corporation, Iselin, NJ), at the manufacturer recommended dosage 5% (w/v). Each plant was treated with a mean volume of 150 mL of spraying solution. All spray applications were supplemented with 0.1% (v/v) Tween 20 and conducted according to good efficacy practice standard operating procedures adjusted for agricultural experiments. Care was taken during the application of foliar sprays to avoid over-spraying non-target trees, covering them with a plastic sheet. The treatment was made in the absence of wind in the morning and kaolin

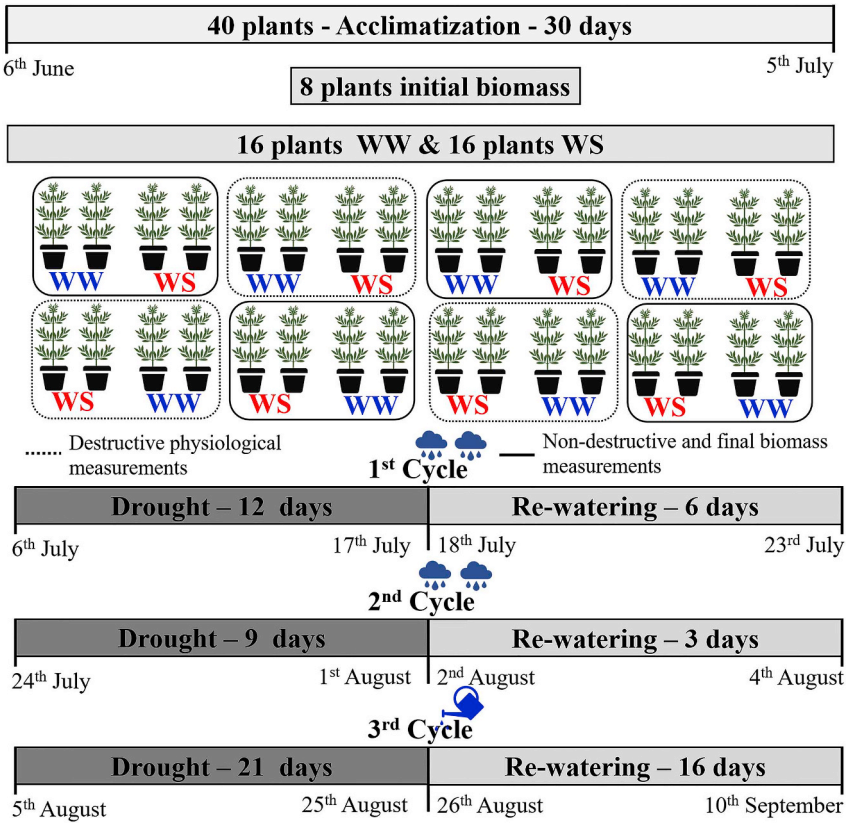

Fig. 1. Schematization of the experiment. Abbreviations: C, control; KL, kaolin treatatment.

were applied when it was necessary due to the rain events. A second application in the same day was done for KL trees, in order to ensure the adhesion uniformity of kaolin clay particles. The applications were made in the beginning of the experiment, 6th July, and after the rain events, 18th July and 2nd August.

Each group of sixteen plants was divided in two subgroups, each one with eight plants. Plants from one subgroup were used for physiological and biochemical destructive measurements, and plants from the other subgroup were used for non-destructive and final biomass assessment. A schematic representation of the experiment is presented in Fig. 1.

All physiological and biochemical measurements at leaf level were measured in healthy, full expanded mature leaves. The daytime leaf gas exchange and leaf relative water content measurements (n = 8) were taken periodically during the three drought-recovery cycles. Nighttime leaf gas exchange, cuticular transpiration and total plant water balance (n = 8) were done at the peak of severest drought period (DP) (3rd cycle). Leaf samples for biochemical analysis (n = 8) were taken at the peak of severest DP (3rd cycle) and eight days after the respective recovery period (RP). Leaves for anatomical tissue measurements (n = 8) were collected at the end of the experiment. For growth, biomass accumulation and whole-plant water use efficiency (n = 8), plants were harvested at the end of the experiment.

2.2. Leaf water status, sclerophylly and structural traits

Leaves detached were immediately placed into air-tight containers and then the following parameters were examined: fresh mass (FM, g); mass at full turgor (TM, g), measured after immersion of leaf petioles in demineralized water for 48 h in the dark at 4 °C; leaf area (LA, cm^2), measured using the WinDias image analysis system (Delta-T Devices Ltd., Cambridge, UK); and dry mass (DM, g), measured after drying at 70 °C to a constant weight. Further, was calculated the relative water content, RWC (%) = (FM – DM)/(TM – DM) x 100, and succulence (mg H_2O cm^{-2}) = (FM – DM)/LA, to characterize leaf water status, and the sclerophylly index, density of foliar tissue (g kg^{-1}) = DM/FM.

For structural analysis, leaves were prepared and analysed as described in Brito et al. (2018). The following variables were determined, thickness of total section (TS), spongy (SP), upper (UPP) and lower (LPP) palisade parenchyma, upper (UE) and lower (LE) epidermis, upper cuticle (UC) and trichome layer (TL).

2.3. Leaf gas exchange and chlorophyll a fluorescence

Leaf gas exchange measurements were performed using a portable IRGA (LCpro+, ADC, Hoddesdon, UK), operating in the open mode. During daylight were performed in the morning (10:00 local time) of summer cloudless days under natural irradiance, and at night in the first hours of the night (22:30–23:30 local time). At night, just before gas exchange quantifications were taken, dew water was removed with an absorbent paper to avoid interferences in gas exchange measurements. The photosynthetic photon flux density (PPFD) and air temperature are provided as supplementary material (supplemental Table 1). Net photosynthetic rate (A_n, μmol CO_2 m^{-2} s^{-1}), daytime (g_{day}, mmol H_2O m^{-2} s^{-1}) and nighttime (g_{night}, mmol H_2O m^{-2} s^{-1}) stomatal conductance, daytime (E_{day}, mmol H_2O m^{-2} h^{-1}) and nighttime (E_{night}, mmol H_2O m^{-2} h^{-1}) transpiration rate, daytime ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a), nighttime respiration rate (R_{night}, μmol CO_2 m^{-2} s^{-1}), and nighttime concentration of CO_2 in intercellular spaces ($C_{i\text{-}night}$, μmol mol^{-1}) were estimated using the equations developed by von Caemmerer and Farquhar (1981). Intrinsic water use efficiency was calculated as the ratio of A_n/g_{day} (μmol mol^{-1}).

Chlorophyll *a* fluorescence parameters were measured in the same leaves and environmental conditions used for gas exchange measurements, with a pulse-amplitude-modulated fluorometer (FMS 2, Hansatech Instruments, Norfolk, England). Prior to the measurements,

a small part of the leaves was dark-adapted for 30 min using dark-adapting leaf-clips. After this period, the minimal fluorescence (F_0) was measured when all photosystem II (PSII) reaction centers are open using a low intensity pulsed measuring light source. The maximal fluorescence (F_m) was measured when all PSII reactions centers are closed during a pulse saturating light (0.7 s pulse of 15000 μmol photons m^{-2} s^{-1} of white light). The difference between these two levels (F_m-F_0) is called variable fluorescence (F_v). Maximum quantum efficiency of PSII was calculated as F_v/F_m = (F_m-F_0)/F_m (Krause and Weis, 1991). Following F_v/F_m estimation, after a 20 s exposure to actinic light (1500 μmol $m^{-2}s^{-1}$), light-adapted steady-state fluorescence yield (F_s) was averaged over 2.5 s, followed by exposure to saturating light (15000 μmol $m^{-2}s^{-1}$) for 0.7 s to establish F'_m. The sample was then shaded for 5 s with a far-red light source to determine F'_0. From these measurements, several fluorescence attributes were calculated according to Bilger and Schreiber (1986) and Genty et al. (1989): photochemical quenching (qP = (F'_m-F_s)/(F'_m-F'_0)), capture efficiency of excitation energy by open PSII reaction centers (F'_v/F'_m = (F'_m-F'_0)/F'_m) and effective quantum efficiency of PSII (ΦPSII = ΔF/F'_m = (F'_m-F_s)/F'_m). Due to a problem in the fluorometer, it was not possible to assess the chlorophyll *a* fluorescence responses in the last monitored date (16(R3)).

2.4. Leaf cuticular transpiration and total plant water balance

In order to discern whether measured values of g_{night} and E_{night} with IRGA were mostly cuticular or stomatal, cuticular water loss ($E_{cuticular}$, mmol m^{-2} s^{-1}) was estimated by the weight loss method, as described by Howard and Donovan (2007). Total plant water balance was accessed based on the gravimetric method. Pot mass changes was monitored during a period of 21 h as reported elsewhere (Brito et al., 2018). From these measurements were estimated the whole-nighttime transpiration ($PE_{w\text{-}night}$, g H_2O m^{-2} n^{-1}) and the whole-daytime transpiration ($PE_{w\text{-}daytime}$, g H_2O m^{-2} $daytime^{-1}$).

2.5. Foliar metabolic assays and oxidative stress markers

For leaf biochemical analysis, the harvested leaves were immediately frozen in liquid nitrogen and stored at −80 °C until be analysed. To express the metabolites by dry mass, a representative sample of each analyzed leaf was evaluated in fresh and after drying at 60 °C until constant weight. Chlorophylls and carotenoids were extracted with acetone/water (80/20, v/v). Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll ($Chl_{(a+b)}$) and Chl_a/Chl_b ratio were determined according to Arnon (1949) and Sesták et al. (1971) and total carotenoids according to Lichtenthaler (1987) and expressed as mg g^{-1}DM. Lycopene and β-carotene were extracted with acetone/hexane (4/6, v/v), determined according to Barros et al. (2011), and expressed as mg g^{-1}DM.

Soluble sugars were extracted according to Irigoyen et al. (1992), by heating the samples in ethanol/water (80/20, v/v) during 1 h, at 80 °C. Then, the soluble fractions were separated from the solid fraction. Starch was extracted by heating the same solid fraction in 30% perchloric acid during 1 h, at 60 °C, according to Osaki et al. (1991). Both SS and St concentration was determined by the anthrone method and expressed as mg g^{-1} DM, using glucose as a standard.

Total soluble proteins were quantified using the method of Bradford (1976), using bovine serum albumin as a standard, and expressed as mg g^{-1}DW. Then, total thiols in soluble proteins extract were assessed according to Ellman (1959), using an extinction coefficient of 13,600 M^{-1} cm^{-1}, and being expressed as nM mg^{-1}DM.

Total phenolics in leaf extracts were quantified following the Folin–Ciocalteu procedure (Singleton and Rossi, 1965) and expressed as mg g^{-1}DM, using gallic acid as a standard. Flavonoids were determined according to Zhishen et al. (1999), using (+)-catechin as a standard, and expressed as mg g^{-1}DM.

Ascorbate was quantified using a method adapted from Klein and Perry (1982), using L-ascorbic acid as a standard, and expressed as mg g^{-1}DM.

Total antioxidant capacity (TAC) based on DPPH-free radical scavenging capacity of leaf extracts was evaluated according to a method adapted from Xu and Chang (2007). Leaf methanolic extracts, and methanol for negative control, were mixed with DPPH methanolic solution (0.1 mM) and left to stand for 30 min in dark at room temperature. The absorbance for the sample (A_{sample}) and negative control ($A_{control}$) was measured at 517 nm against methanol blank. The percent of DPPH radical reduction was calculated as follows = 100 x ($A_{control}$ − A_{sample})/$A_{control}$. The free radical scavenging activity was expressed as μM of Trolox equivalents (TE) per g^{-1}DM (TE = (% DPPH radical reduction/a)), where *a* is the slope of the standard curve (y = ax).

Total reactive oxygen species (ROS) were determined with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma–Aldrich,Germany) (Kong et al., 2013). A 25 mM solution was prepared in dimethyl sulphoxide for pending use. Twenty microliters of each sample were loaded into a small well ELISA plate containing 0.2 ml of PBS buffer (pH 7.4) and 12 μM of DCFH-DA and incubated for 20 min at 25°∘C. Fluorescence was measured at 485 nm and 530 nm (excitation and emission wavelength, respectively), in a CARY 50 Bio (Eclipse, Australia) every 15 min until 60 min after the incubation. 2′,7′,-dichlorofluorescein was used to obtained a calibration curve. Results were expressed as nM DCF g^{-1}DM.

H_2O_2 concentration were determined using a method described by Junglee et al. (2014), with some modifications. The absorbance was measured at 350 nm and H_2O_2 was used to obtain a calibration curve. Results were expressed in μM g^{-1}DM.

Leaf electrolyte leakage was measured as an indicator of cell membrane permeability, following a procedure adapted from Mena-Petite et al. (2001) and described in Brito et al. (2018).

2.6. Biomass accumulation and whole-plant water use efficiency

Plants were harvested and total leaf area (WinDias image analysis system (Delta-T Devices Ltd., Cambridge, UK) and the dry weight of aboveground and belowground organs, after drying in a force-draft oven at 70 °C to a constant weight, were determined. Based on these data were determined the mean net assimilation rate (NAR, rate of biomass gain per leaf area), using the equation proposed by Hunt (1978), and the relative alleviation index (RAI), estimated according to Gupta et al. (1995).

Water use efficiency of biomass production (WUE_{WP}, g kg^{-1}) was determined, for each plant, by dividing total dry matter production by the cumulative amount of water used throughout the growing season. Total dry matter included the oven-dried leaves, stems and roots.

2.7. Statistical analysis

All statistical calculations were performed using the software program SPSS for Windows (v. 22). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences were evaluated by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test ($P < 0.05$). For statistical analysis of RWC, EL and BI arcsine transformation was performed in percentage data. The relationships between R_{night} and E_{night}, R_{night} and $C_{i\text{-}night}$, and g_{night} and $C_{i\text{-}night}$ was analyzed by the Pearson correlation test and significance was set at $P < 0.05$.

3. Results and discussion

3.1. Physiological and structural traits modulated by kaolin

Kaolin has been used as a tool for saving water and to improve crop

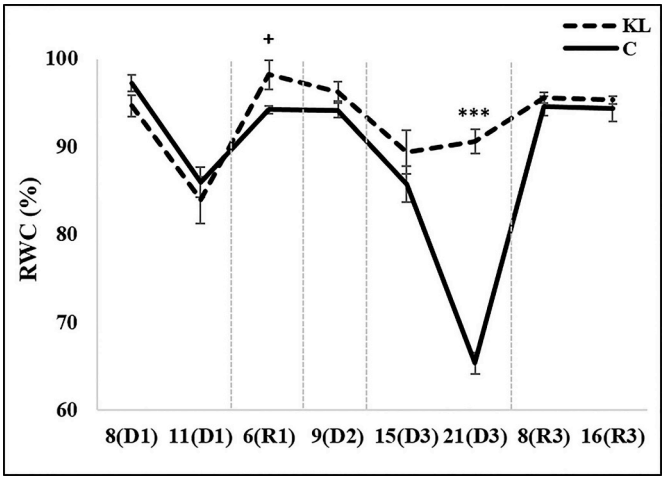

Fig. 2. Changes of leaf relative water content (RWC) during days of drought (D1, D2, D3) and recovery (R1, R3) in each cycle, in leaves of control (C) and kaolin (KL) plants. Each point is average and vertical bars represent the S.E. (n = 8). Significance: +0.1 > P > 0.05, ***P < 0.001.

performance, leading to lower temperature of sprayed organs (Segura-Monroy et al., 2015; Dinis et al., 2018), as the particle film reflects PAR, UV and IR radiation. The reduction of leaf-to-air vapor pressure deficit ($VPDl_{eaf\text{-}air}$) is also a regular effect (Jifon and Syvertsen, 2003; Rosati et al., 2006). Consequently, KL contributed to attenuate the drought-induced decline of RWC during the 3rd DP (Fig. 2) and to increase leaves succulence (Table 1). Hence, our data demonstrate the effectiveness of this treatment in water-saving under severe conditions, as observed in other studies (Boari et al., 2015; Denaxa et al., 2012; Nanos, 2015). This ability to maintain turgid leaves in drought environments have several physiological advantages, allowing the maintenance of turgor dependent processes, such as growth, stomatal activity and photosynthesis (Mullan and Pietragalla, 2011).

In a closely association with plant water status (Fig. 2), kaolin film had implications on leaf sclerophylly and structural traits, as KL leaves displayed lower density and thickness (Table 1 and Fig. 3). KL plants exhibited thinner leaves, due to lower UC and UPP, that in turns contributed to reduce PP/SP ratio, which indicates a less compact arrangement of mesophyll cells (Bacelar et al., 2004). Interestingly, all these changes are typical plant responses to shade. Similar results for leaf density (Denaxa et al., 2012), leaf thickness (Segura-Monroy et al., 2015), and other shade-related characteristics (Nanos, 2015) were reported previously in response to kaolin application. On the other hand, no significant differences were observed for both epidermis, SP, LPP and TL (Table 1).

Table 1
Leaf sclerophylly and structural traits of control (C) and kaolin (KL) plants. Succulence (mg H_2O cm^{-2}), density (g kg^{-1}), and leaf tissues thickness (μm; total section, LT, upper cuticle, UC, upper epidermis, UE, upper palisade parenchyma, UPP, spongy parenchyma, SP, lower palisade parenchyma, LPP, lower epidermis, LE, palisade/spongy parenchyma ratio, PP/SP and trichome layer, TL).

	C	KL	Sig.
Succulence	18.97 ± 1.19	27.9 ± 0.88	**
Density	576.7 ± 6.5	495.7 ± 7.2	*
LT	512.5 ± 9.3	473.8 ± 12.1	*
UC	7.53 ± 0.24	6.61 ± 0.24	*
UE	16.84 ± 0.53	18.03 ± 0.64	n.s.
UPP	172.6 ± 9.77	138.9 ± 4.09	*
SP	221.7 ± 3.8	214.7 ± 6.09	n.s.
LPP	29.54 ± 1.09	29.20 ± 1.92	n.s.
LE	16.36 ± 0.28	16.45 ± 0.52	n.s.
PP/SP	0.960 ± 0.042	0.825 ± 0.029	*
TL	47.86 ± 2.10	49.81 ± 3.80	n.s.

Values are means ± SE (n = 8). Significance: n.s., not significant, *P < 0.05, **P < 0.01.

Olive leaf stomata respond strongly to leaf RWC, as well to higher vapor pressure deficit (VPD). So, as KL may lower $VPD_{leaf\text{-}air}$, the sprayed plants were able to keep higher g_{day} and, thus, A_n and E_{day} during the DPs (Fig. 4 A, B, C). Similar effects of kaolin on leaf gas exchange variables were reported previously in different plant species (Jifon and Syvertsen, 2003; Glenn, 2009; Boari et al., 2015), including olive tree (Denaxa et al., 2012; Nanos, 2015). Nonetheless, KL influence was higher on g_{day} than on A_n, contributing to lower A_n/g_{day} during the 2nd and 3rd DPs (Fig. 4D).

Despite the predominance of stomatal effects, also corroborated by C_i/C_a data (Fig. 4D), KL influenced photosynthetic activity by the preservation of photosynthetic machinery, due to the reduction of heat load and irradiation levels, as demonstrated by Dinis et al. (2018). In this study, KL spray was effective in the photochemistry processes protection (Fig. 5), as demonstrated by the lower F_o until the middle of the 3rd DP (Fig. 5A), the higher F_v/F_m, until the 2nd DP (Fig. 5B), and the general trend to superior ΦPSII, with the exception at the peak of the 3rd DP (Fig. 5C). Nevertheless, KL did not interfere with the proportion of open PSII reaction centers during drought, as demonstrated by the absence of significant differences on qP (Fig. 5D) (Baker, 2008). As qP had a slight influence on ΦPSII, the capacity of KL plants to keep higher ΦPSII was more related to F'_v/F'_m response (Fig. 5E), which indicates a reduced loss of excitation energy by thermal dissipation, which could compete with its transfer to PSII reaction centers (Baker, 2008). A positive kaolin influence on the light-dependent reactions of photosynthesis was also described in other studies, with higher effectiveness around midday period (Jifon and Syvertsen, 2003; Segura-Monroy et al., 2015).

Regarding recovery events, RWC values showed a prompt recovery (Fig. 2). This is a typical response of olive tree that through a conservative behaviour rapidly restore water status after stress relief (Perez-Martin et al., 2014). On the other hand, although only significant values of g_{day} were found at the 1st RP, g_{day} showed a reverse pattern with respect to the DP (Fig. 4B), demonstrating that KL trees had a delay on g_{day} recovery after rewatering. Under optimal hydration, plants increase transpiration and the resulted evaporative cooling effect lower $VPD_{leaf\text{-}air}$. Under these conditions the low light intensity promoted by kaolin may prevail leading to a reduction of g_{day} (Gregoriou et al., 2007). Similar findings were reported previously (Roussos et al., 2010; Denaxa et al., 2012; Shellie and King, 2013; Boari et al., 2015). As a consequence of lower g_{day}, KL also retarded the A_n recovery (Fig. 4A). In addition, both the partial shade-related characteristics developed by KL leaves and the reduction in solar irradiation limited the recovery of photosynthetic activity. It is important to highlight that the year of the study had an atypical summer season with enhanced cloudy days than usual, meaning that with low PPFD the shade effect is undesirable for KL effectiveness. Denaxa et al. (2012) also showed that during the time of the day when light intensity is lower, kaolin limited A_n of irrigated olive trees, while no such effect was recorded at midday hours.

Regarding physiological responses during nighttime, this study demonstrated that g_{night} and, consequently, E_{night} respond to kaolin application, being the leaf water losses during the first hours of night repressed by KL (Table 2). Meanwhile, in C leaves E_{night} was highly far above $E_{cuticular}$, demonstrating that E_{night} is mainly due to stomatal losses, while in KL leaves, the lower E_{night} was more related with $E_{cuticular}$, as a negligible water loss by stomata was observed (Table 2). Moreover, the differences in $E_{cuticular}$ were related with the histological analysis, as KL leaves presented thinner cuticle layer (Table 1). The higher nighttime transpiration of C plants was also confirmed gravimetrically, while during the daytime period was observed a clear opposite tendency (Table 2), in a closely association with g_{day} values (Fig. 4B). It has been demonstrated that nighttime transpiration can

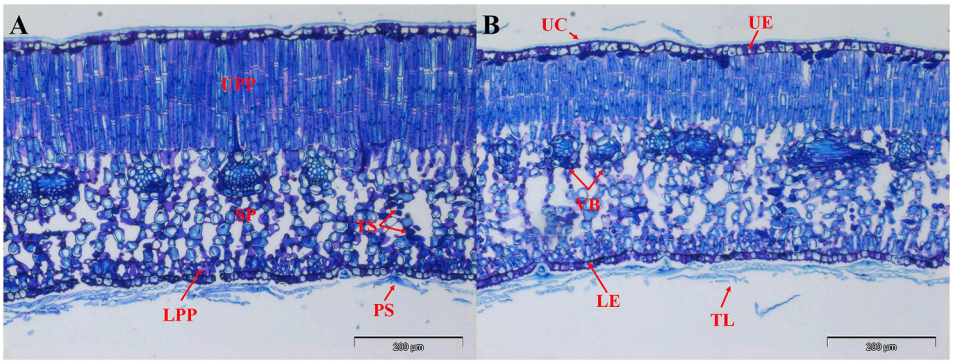

Fig. 3. Light microscopy images of olive leaf transversal sections stained with toluidine blue. Control (A) and Kaolin (B) treatments. Abbreviations: UC = upper cuticle; UE = upper epidermis UPP = upper palisade parenchyma; SP = spongy parenchyma; LPP = lower palisade parenchyma; VB = vascular bundles; TS = trichosclereids; LE = lower epidermis; TL = trichome layer; PS = peltate scales. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

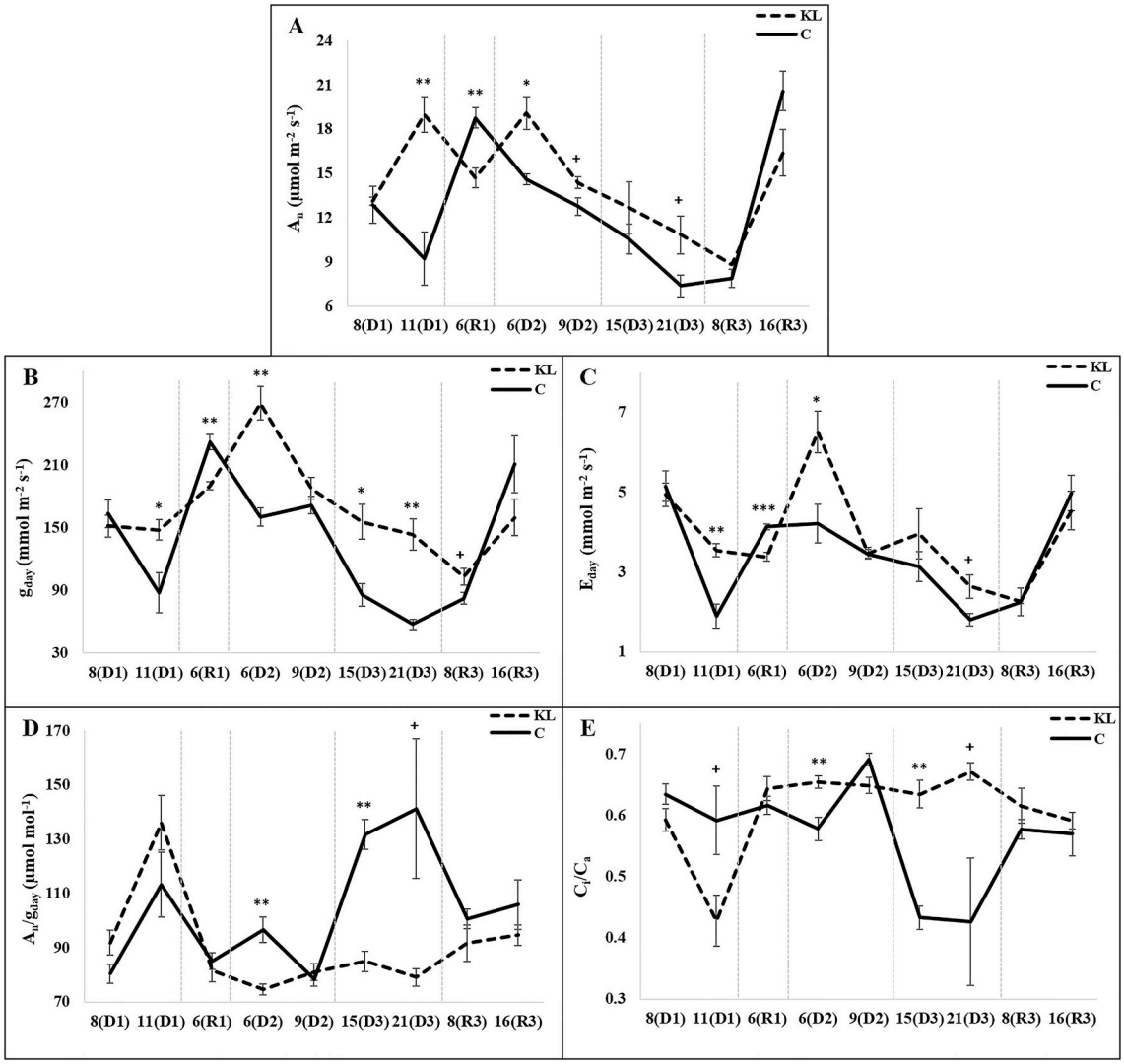

Fig. 4. Changes of net photosynthetic rate (A_n, A), stomatal conductance (g_{day}, B), transpiration rate (E_{day}, C), intrinsic water use efficiency (A_n/g_{day}, D) and ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a, E) during days of drought (D1, D2, D3) and recovery (R1, R3) in each cycle, in leaves of control (C) and kaolin (KL) plants. Each point is average and vertical bars represent the S.E. (n = 8). Significance: +0.1 > P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

account for around 10% of daily transpiration (Escalona et al., 2013; Medrano et al., 2015), reducing water use efficiency at whole-plant scale (Medrano et al., 2015). Our data indicate that kaolin contributed to reduce the relative nighttime transpiration, as in KL plants $PE_{w\text{-}night}$ account to 8.6% of whole-day transpiration, against 21% in C plants (Table 2). However, since nighttime transpiration can lead to a reduction in WUE raises the question, why control stressed-plants risk to lose water when there is no opportunity for carbon gain? There is no consistent knowledge about g_{night} and much factors can act isolated or in combination. E_{night} may lower leaf temperature by evaporative cooling, thereby decreasing carbon losses through R_{night} (Coupel-Ledru et al., 2016), a hypothesis supported in our study by the significant negative correlation between E_{night} and R_{night} ($r = -0.723$; $P < 0.01$). This response takes especially importance in the first hours of darkness, once

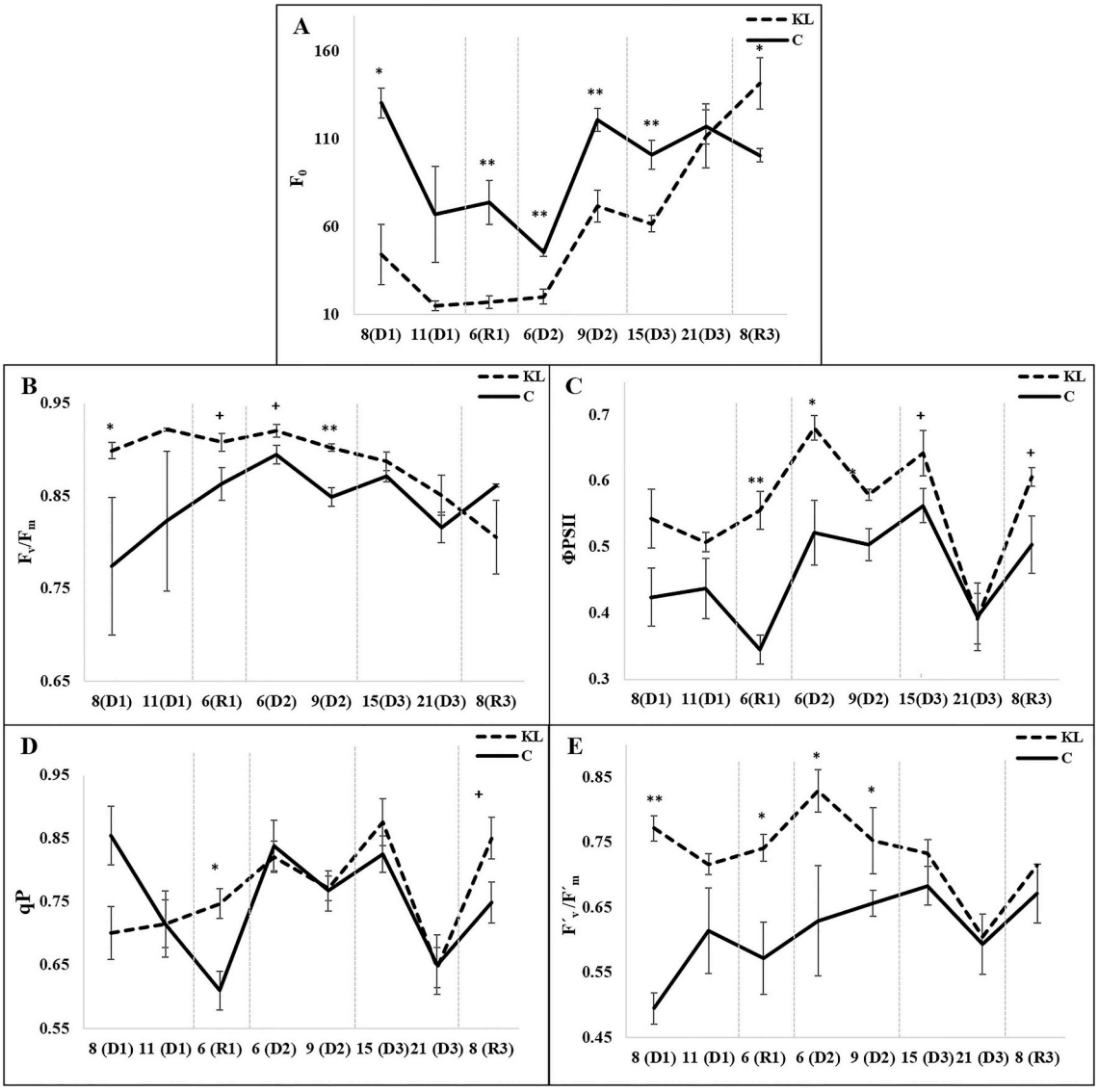

Fig. 5. Changes in minimal fluorescence emitted from dark adapted leaves (F_0, A), maximum (F_v/F_m, B) and effective (ΦPSII, C) quantum efficiency of PSII, photochemical quenching (qP, D), and capture efficiency of excitation energy by open PSII reaction centers (F'_v/F'_m, E) during days of drought (D1, D2, D3) and recovery (R1, R3) in each cycle, in leaves of control (C) and kaolin (KL) plants. Each point is average and vertical bars represent the S.E. (n = 8). Significance: +0.1 > P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

Table 2
Leaf nighttime stomatal conductance (g_{night}, mmol m^{-2} s^{-1}), nighttime transpiration rate (E_{night}, mmol m^{-2} s^{-1}), cuticular transpiration ($E_{cuticular}$, mmol m^{-2} s^{-1}), nighttime respiration rate (R_{night}, μmol m^{-2} s^{-1}) and nighttime intercellular CO_2 concentration ($C_{i\text{-}night}$, μmol mol^{-1}) and whole-plant transpiration during the nighttime ($PE_{w\text{-}night}$, g H_2O m^{-2} n^{-1}) and daytime ($PE_{w\text{-}daytime}$, g H_2O m^{-2} $daytime^{-1}$) of control (C) and kaolin (KL) plants.

	C	KL	Sig.
g_{night}	17.65 ± 1.15	5.84 ± 0.75	***
E_{night}	0.273 ± 0.014	0.098 ± 0.003	***
$E_{cuticular}$	0.046 ± 0.003	0.095 ± 0.005	***
R_{night}	1.20 ± 0.36	1.90 ± 0.07	*
$C_{i\text{-}night}$	515.6 ± 26.0	981.6 ± 84.9	**
$PE_{w\text{-}night}$	79.4 ± 14.7	39.1 ± 6.1	*
$PE_{w\text{-}daytime}$	298.8 ± 39.5	413.4 ± 42.1	+

Values are means ± SE (n = 8). Significance: +0.1 > P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001).

Table 3
Foliar pigments concentrations (mg g^{-1} DW) of control (C) and kaolin (KL) at the peak of severest drought period (21(D3)) and 8 days after rewatering (8(R3)). Total chlorophyll ($Chl_{(a+b)}$), chlorophyll a/b ratio (Chl_a/Chl_b), total carotenoids, $Chl_{(a+b)}$/Carotenoids ratio, lycopen and β-Carotene.

		C	KL	Sig.
$Chl_{(a+b)}$	**21(D3)**	2.80 ± 0.13	3.21 ± 0.05	*
	8(R3)	3.36 ± 0.06	3.31 ± 0.07	**n.s.**
Chl_a/Chl_b	**21(D3)**	3.11 ± 0.01	3.04 ± 0.02	+
	8(R3)	2.97 ± 0.015	3.12 ± 0.02	**n.s.**
Carotenoids	**21(D3)**	0.656 ± 0.032	0.707 ± 0.012	**n.s.**
	8(R3)	0.720 ± 0.023	0.717 ± 0.018	**n.s.**
$Chl_{(a+b)}$/Carotenoids	**21(D3)**	4.48 ± 0.032	4.77 ± 0.042	**
	8(R3)	4.91 ± 0.25	4.85 ± 0.05	**n.s.**
Lycopene	**21(D3)**	1.21 ± 0.03	1.30 ± 0.04	**n.s.**
	8(R3)	1.35 ± 0.05	1.10 ± 0.04	*
β-Carotene	**21(D3)**	0.554 ± 0.012	0.534 ± 0.010	**n.s.**
	8(R3)	0.637 ± 0.013	0.564 ± 0.026	*

Values are means ± SE (n = 8). Significance: n.s., not significant, +0.1 > P > 0.05, *P < 0.05, **P < 0.01.

temperatures are usually higher. In addition, a reduced accumulation of $C_{i\text{-}night}$ (Table 2), due to the reduced R_{night} ($r = 0.789$; $P < 0.001$), may also have contributed to stomatal open (Escalona et al., 2013), as attested by the negative correlation between $C_{i\text{-}night}$ and g_{night} ($r = -0.945$; $P < 0.001$). Thus, this study confirms that the olive tree capacity to maintain low R_{night} rates during more stressful conditions, allows this species to allocate more assimilates for biomass accumulation and, consequently, for growth (Varone and Gratani, 2015), despite the higher leaf density and thickness (Table 1) and decline in A_n (Fig. 4A).

3.2. Changes in non-structural carbohydrates and oxidative stress markers induced by kaolin

Environmental stresses perturb the equilibrium between the production and scavenging of ROS, inducing damage to several biomolecules (Sharma et al., 2012). Nevertheless, only few studies reported data about cellular metabolic dynamics in relation to kaolin application, and those were mainly related with photosynthetic pigment analysis. Our data showed higher concentration of chlorophylls in KL plants (Table 3), a sign of lower oxidative stress, allowing that these plants can use light energy more efficiently. Chlorophylls preservation associated to kaolin application had also been reported in other studies (Nanos, 2015; Segura-Monroy et al., 2015). In addition, KL leaves exhibited lower Chl_a/Chl_b ratio during drought (Table 3), as in other works (Nanos, 2015; Shellie and King, 2013). Lower Chl_a/Chl_b ratio is a typical feature of low-light adapted leaves, indicating that photosystems have larger antenna sizes at the expense of reaction centers pigment proteins, to enhance their ability to capture and utilize photon energy (Gregoriou et al., 2007; Lichtenthaler et al., 2007). Furthermore, as total carotenoids concentration was not significant different between treatments, $Chl_{(a+b)}$/Carotenoids ratio was significantly higher in KL leaves (Table 3), also a shade-related trait (Lichtenthaler et al., 2007). Since carotenoids play an important role in photoprotection, scavenging ROS and releasing the excess energy by thermal dissipation via xanthophyll cycle (Lisar et al., 2012; Sharma et al., 2012), the higher $Chl_{(a+b)}$/Carotenoids ratio observed in KL leaves indicates a lower need for photoprotection of chlorophylls. Meanwhile, the concentration of chlorophylls was restored immediately after rewatering (Table 3), probably as a result of *de novo* synthesis of chlorophylls, as in the study of Tognetti et al. (1995). The maintenance of high chlorophyll concentration during drought was noted to contribute to a rapid recovery of photosynthesis (Chen et al., 2016). However, we found no such influence on A_n recovery of KL leaves, meaning that photosynthetic recovery of KL trees was mainly limited by stomata. Although the concentration of total carotenoids was not affected by rewatering, the relative proportion of individuals changed, as revealed by higher investment on lycopene and β-carotene by control plants (Table 3). Lycopene is the starting compound of various end group modifications that produces a large variety of carotenoids, such as β-carotene, which display the ability to quench triplet chlorophyll and singlet oxygen (Domonkos et al., 2013), preventing photosynthetic apparatus damage.

A fraction of carbon acquired via photosynthesis is retained in the form of non-structural carbohydrates. At the peak of the 3rd DP, KL leaves presented higher and lower concentrations of soluble sugars and starch, respectively (Table 4). Upon rewatering, no significant differences were observed in soluble sugars levels, as a result of the higher increase of soluble sugars in C plants. On the other hand, St concentration remained higher in C plants, in spite of the higher decrease during the RP, suggesting a mobilization of these reserves. These results are consistent with a dual view of non-structural carbohydrates function. Soluble sugars are sources of carbon for maintenance and regrowth during recovery and may act as osmoprotectants (Chaves et al., 2002). Since the primary function of compatible solutes is to prevent water loss, by maintaining cell turgor and the gradient for water uptake into cells (Lisar et al., 2012) might contributed to the higher RWC and stomatal opening exhibited by KL leaves (Figs. 2 and 4B), Moreover, compatible solutes are also involved in detoxification of ROS and stabilization of cellular macromolecules structures (Lisar et al., 2012). On the other hand, starch acts mostly as a reservoir of carbon for future use, depending on the source-sink dynamics concept. The higher starch contents in C leaves, also verified earlier in olive tree under severe drought conditions (Bacelar et al., 2006), in spite of lower A_n, suggest that carbon was not translocated out of the leaves, reflecting an excess supply relative to demand. Conversely, the lower starch concentrations in KL leaves may be linked to tissue osmotic adjustment, as one of the main sources of osmolytes are the starch reserves, which supply soluble sugars. Although it is usual an increased demand of non-structural carbohydrates to rapid recovery of physiological activities and growth after rewatering (Da costa and Huang, 2006), this is not the general picture of our data (Table 4). To illustrate, was observed an increase of soluble sugars during rehydration, namely in C plants, in line with a tendency to lower starch content. These results showed that, following rehydration, olive trees divert a higher proportion of the assimilated carbon into soluble sugar export for plant growth, and less to temporary storage, as starch, a common response of fast growing species (Liu et al., 2017). Thus, we may assume that growth of trees under drought and rewatering cycles are positively associated with higher soluble sugars/starch ratio, particularly clear in KL plants, and not to higher non-structural carbohydrates concentration.

Kaolin application contributed to keep higher total soluble proteins concentrations (Table 4), possibly due to the better water status and the plausible reduced leaf temperature. Indeed, a reduction in leaf soluble proteins concentration was already reported in plants subjected to drought (Bacelar et al., 2007) and heat (Gulen and Eris, 2004). The accumulation of ROS under stressful conditions induces oxidative damage in proteins (Farooq et al., 2009), and high temperatures also causes protein denaturation and aggregation (Hasanuzzaman et al., 2013). One of the more susceptible targets in proteins are thiol groups, with relevant role in signalling in a range of physiological processes, but when suffer from irreversible oxidation can seriously damage proteins (Chouchani et al., 2011). Thiol groups are usually affected by water deficit, as already reported in olive trees (Bacelar et al., 2006, 2007), but kaolin contributed to keep higher total thiols levels during the DP (Table 4). After stress relief, both treatments increased soluble proteins contents (Table 4), suggesting that proteins were suppressed due to water deficit in both treatments, as in Flexas et al. (2006), although in a different extent. In addition to the higher soluble proteins concentration upon rewatering, KL leaves also presented higher increase from the peak of drought stress, with an enhancement of 52% against 35% exhibited by C leaves. Moreover, only the last leaves increased total thiols levels (Table 4) in response to rewatering, suggesting, at some extent, a reversible oxidation during drought and possible effects in signalling pathways.

As a result of the worst physiological status, reported before, C plants needed to invest more resources in antioxidant defences during drought, as demonstrated by ascorbate and total phenolic concentrations and by total antioxidant activity data (Table 4). Ascorbate, besides to directly scavenge ROS, is also a substrate to ascorbate peroxidase that use it as specific electron donor to reduce H_2O_2 to H_2O (Mattos and Moretti, 2015; Sharma et al., 2012). Moreover, in chloroplasts, ascorbate acts as a cofactor of violaxantin de-epoxidase, thus sustaining dissipation of excess excitation energy (Mattos and Moretti, 2015). Phenolic compounds, to which flavonoids belong, possess the ideal chemistry for free radical scavenging because of their strong capacity to donate electrons or hydrogen atoms, acting actively as plant antioxidants (Mattos and Moretti, 2015). It is noteworthy that total phenolics increase was largely associated with the rise of flavonoids (Table 4), which, in addiction to be efficient scavengers, serve multiple functions in photoprotection under high sunlight conditions and act as UV-B screening (Agati et al., 2013). Another mechanism underlying the antioxidant properties of phenolics is the ability of flavonoids to alter

Table 4
Foliar metabolite concentrations of control (C) and kaolin (KL) at the peak of severest drought period (21(D3)) and 8 days after rewatering (8(R3)). Soluble sugars (mg g^{-1}DW), starch (mg g^{-1}DW), soluble proteins (mg g^{-1} DW), total thiols (nmol mg^{-1} DW), total phenolics (mg g^{-1} DW), flavonoids (mg g^{-1} DW), ascorbate (mg g^{-1} DW), reactive oxygen species (ROS, nmol g^{-1} DW) and hydrogen peroxide (H_2O_2, μmol g^{-1} DW) concentrations, total antioxidant capacity (TAC, μmol TE g^{-1} DW), and electrolyte leakage (%).

		C	KL	Sig.
Soluble Sugars	**21(D3)**	209.0 ± 6.1	230.9 ± 6.6	*
	8(R3)	242.5 ± 14.7	254.0 ± 14.8	**n.s.**
Starch	**21(D3)**	64.35 ± 5.80	41.37 ± 3.50	**
	8(R3)	53.29 ± 6.64	35.31 ± 2.42	*
Soluble proteins	**21(D3)**	2.74 ± 0.14	4.59 ± 0.30	**
	8(R3)	3.72 ± 0.13	7.00 ± 0.35	***
Total thiols	**21(D3)**	1.04 ± 0.07	1.27 ± 0.05	*
	8(R3)	1.15 ± 0.04	1.28 ± 0.07	**n.s.**
Total phenolics	**21(D3)**	44.54 ± 0.30	42.07 ± 0.39	**
	8(R3)	44.00 ± 0.39	47.13 ± 0.30	***
Flavonoids	**21(D3)**	23.73 ± 0.25	18.64 ± 0.28	***
	8(R3)	18.47 ± 0.40	19.53 ± 0.22	*
Ascorbate	**21(D3)**	1.68 ± 0.05	1.36 ± 0.02	**
	8(R3)	2.27 ± 0.04	2.62 ± 0.03	***
TAC	**21(D3)**	163.4 ± 1.2	152.6 ± 3.4	*
	8(R3)	199.1 ± 4.2	195.1 ± 3.9	**n.s.**
ROS	**21(D3)**	0.443 ± 0.020	0.554 ± 0.043	**
	8(R3)	0.588 ± 0.041	0.511 ± 0.053	**n.s.**
H_2O_2	**21(D3)**	12.48 ± 0.15	19.53 ± 0.23	***
	8(R3)	18.74 ± 0.59	24.18 ± 0.35	***
Electrolyte leakage	**21(D3)**	27.56 ± 0.56	18.54 ± 3.41	*
	8(R3)	24.05 ± 1.78	19.87 ± 0.89	+

Values are means ± SE (n = 8). Significance: n.s., not significant, +0.1 > P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001).

peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora et al., 2000). Interestingly, the concentrations of total phenolics, flavonoids and ascorbate increased with rewatering, namely in KL leaves, being reversed the previous pattern, as those concentrations were now higher in KL trees (Table 4). This suggest a rearrangement of many metabolic pathways, in order to a better balance between repair of drought-induced damages, activation of a battery of plant defences and stimulation of plant growth processes. Furthermore, kaolin contributed to maintain lower cellular membrane dysfunction, as showed by the inferior electrolyte leakage during drought (Table 4). Upon rewatering, C leaves had less leakage of ions, but still presented a tendency to higher electrolyte leakage than KL leaves (Table 4).

Despite KL leaves had lower signals of oxidative damage, they exhibited higher concentrations of both total ROS and H_2O_2 during drought (Table 4). Conversely, Dinis et al. (2016) observed inferior accumulation of ROS in response to kaolin application in grapevines leaves. ROS are an inevitable by-product of aerobic metabolism, damaging biomolecules if were excessively produced (Sharma et al., 2012). However, at low/moderate levels they act as second messengers in a variety of cellular processes, including conferment of tolerance to environmental stresses (Sharma et al., 2012), specially H_2O_2 due to its long half-life and the ability to cross cellular membranes (Petrov and Van Breusegem, 2012). Because of the multifunctional roles of ROS, it is necessary for the cells to control their levels tightly to avoid any oxidative injury, but not eliminating them completely (Sharma et al., 2012). Taken together, these results suggest that ROS levels in KL leaves may still not be excessively harmful. In addition, in C plants, the oxidative damages already caused might triggered the antioxidative responses, that effectively reduced the ROS levels, to avoid extra oxidative damages, showing that C plants are not severely stressed. Moreover, the increase in total ROS in C leaves after stress relief, linked with the increase of H_2O_2 in both treatments (Table 4) suggest that these levels might occur due to normal plant metabolism.

Table 5
Total leaf area (TLA, cm^2 $plant^{-1}$), total biomass increase (BI, %), whole-plant water use efficiency (WUE_{WP}, g kg^{-1}), relative alleviation index (RAI) and net assimilation rate (NAR, g m^2 day^{-1}) of control (C) and kaolin (KL) plants at the end of the experiment.

	C	KL	Sig.
TLA	1078.1 ± 44.8	1208.2 ± 76.3	**n.s.**
BI	26.67 ± 5.84	37.22 ± 9.60	**n.s.**
WUE_{WP}	4.15 ± 0.91	5.78 ± 1.49	**n.s.**
RAI	100.0 ± 4.4	108.0 ± 7.3	**n.s.**
NAR	3.19 ± 0.67	4.24 ± 1.08	**n.s.**

Values are means ± SE (n = 8). Significance: n.s., not significant.

3.3. Growth, biomass accumulation and water use efficiency under kaolin application

In general, is recurrent to find a positive effect of kaolin application on plant growth and/or yield under drought stress conditions (Roussos et al., 2010; Segura-Monroy et al., 2015). However, in the present study, in spite of the lower oxidative injury and the best physiological performance observed during the DPs in KL plants, leaf area, net assimilation rate, total biomass increase and water use efficiency for biomass production were not significant different between treatments (Table 5), which were associated with the negative A_n responses after rewatering (Fig. 4A). Moreover, the differences were also mitigated by the high number of days (25 out of 66) of the rehydration periods, given the unusual climatic conditions. Nonetheless, there was a tendency to all those variables being higher in KL plants, contributing to an increase of 8% on RAI.

The negative influence of kaolin in leaf A_n/g_{day} during some drought periods of the experiment (Fig. 4D) was not traduced in WUE_{WP}, that presented a slight tendency to be higher in KL trees (Table 5). In fact, A_n/g_{day} and WUE_{WP} cannot be always strictly related, due to spatial and temporal variations, since the first denotes responses of individual leaves at specific environmental conditions, while the second represents the whole-plant carbon and biomass acquisition per unit of water used along all the growth season, integrating other physiological processes like respiration and night transpiration processes (Medrano et al., 2015). A positive influence of kaolin application on WUE_{WP} was described in cape gooseberry by Segura-Monroy et al. (2015).

4. Conclusions

Kaolin is suitable for improving water status and preserving cellular function during the most stressful periods, reducing investment costs in extra repair damages during recovery periods. Kaolin is appropriate to alleviate stomatal and non-stomatal limitations to photosynthesis under water shortage conditions, allowing a better photosynthetic activity during the more severe drought events. Nevertheless, the shaded effect conferred by kaolin interfere with gas exchange restauration after stress relief and also with photosynthetic activity under low light conditions. Kaolin mitigate oxidative stress during drought episodes, reducing the investment in antioxidant defences, and allow a better capacity to restore metabolic functions upon rewatering. Under the present conditions, kaolin did not influence whole-plant water use efficiency and plant biomass accumulation. Taken together, the present results clearly demonstrate the complexity of plant responses to kaolin-induced microclimate changes, which vary depending on the respective biological and environmental conditions. Nevertheless, kaolin may have beneficial effects on realistic field conditions under the prevalence of sunny days, in trees with higher dense canopies, benefiting from the light redistribution within the canopy, and in semi-arid areas, where is recurrent to find groves under rainfed conditions where the incident PAR usually exceeds the capacity of plant use.

Authors contribution

Cátia Brito was responsible for maintaining field experiment, collection and data analysis and manuscript writing. Lia-Tânia Dinis and José Moutinho-Pereira collaborated in data collection, critical review and final approval of the manuscript. Helena Ferreira, Luis Rocha and Ivo Pavia collaborated in data collection. Carlos Correia was responsible for design the experiments, data collection, critical review and final approval of the manuscript.

Acknowledgments

Doctoral fellowship under the Doctoral Program “Agricultural Production Chains – from fork to farm” (PD/00122/2012) provided by the FCT-Portuguese Foundation for Science and Technology to C. Brito (PD/BD/52543/2014). Institution CITAB, for its financial support through the European Investment Funds by FEDER/COMPETE/POCI–Operational Competitiveness and Internationalization Program, under Project POCI-01-0145-FEDER-006958 and National Funds by FCT - Portuguese Foundation for Science and Technology, under the project UID/AGR/04033/2013.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.10.028.

References

Agati, G., Brunetti, C., Di Ferdinando, M., Ferrini, F., Pollastri, S., Tattini, M., 2013. Functional roles of flavonoids in photoprotection: new evidence, lessons from the past. Plant Physiol. Biochem. 72, 35–45. https://doi.org/10.1016/j.plaphy.2013.03.014.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta* vulgaris. Plant Physiol. 24, 1–15. https://doi.org/10.1104/pp.24.1.1.

Arora, A., Byrem, T.M., Nair, M.G., Strasburg, G.M., 2000. Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. Arch. Biochem. Biophys. 373, 102–109. https://doi.org/10.1006/abbi.1999.1525.

Bacelar, E.A., Correia, C.M., Moutinho-Pereira, J.M., Goncalves, B.C., Lopes, J.I., Torres-Pereira, J.M., 2004. Sclerophylly and leaf anatomical traits of five field-grown olive cultivars growing under drought conditions. Tree Physiol. 24, 233–239.

Bacelar, E.A., Santos, D.L., Moutinho-Pereira, J.M., Gonçalves, B.C., Ferreira, H.F., Correia, C.M., 2006. Immediate responses and adaptative strategies of three olive cultivars under contrasting water availability regimes: changes on structure and chemical composition of foliage and oxidative damage. Plant Sci. 170, 596–605. https://doi.org/10.1016/j.plantsci.2005.10.014.

Bacelar, E.A., Santos, D.L., Moutinho-Pereira, J.M., Lopes, J.I., Gonçalves, B.C., Ferreira, T.C., Correia, C.M., 2007. Physiological behaviour, oxidative damage and anti-oxidative protection of olive trees grown under different irrigation regimes. Plant Soil 292, 1–12. https://doi.org/10.1007/s11104-006-9088-1.

Baker, N.R., 2008. Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. Annu. Ver. Plant Biol. 59, 89–113. https://doi.org/10.1146/annurev.arplant.59.032607.092759.

Barros, L., Cabrita, L., Boas, M.V., Carvalho, A.M., Ferreira, I.C.F.R., 2011. Chemical, biochemical and electrochemical assays to evaluate phytochemicals and antioxidant activity of wild plants. Food Chem. 127, 1600–1608. https://doi.org/10.1016/j.foodchem.2011.02.024.

Bilger, W., Schreiber, U., 1986. Energy-dependent quenching of dark-level chlorophyll fluorescence in intact leaves. Photosynth. Res. 10, 303–308. https://doi.org/10.1007/BF00118295.

Boari, F., Donadio, A., Schiattone, M.I., Cantore, V., 2015. Particle film technology: a supplemental tool to save water. Agric. Water Manag. 147, 154–162. https://doi.org/10.1016/j.agwat.2014.07.014.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brito, C., Dinis, L.-T., Ferreira, H., Moutinho-Pereira, J., Correia, C., 2018. The role of nighttime water balance on *Olea europaea* plants subjected to contrasting water regimes. J. Plant Physiol. 226, 56–63. https://doi.org/10.1016/j.jplph.2018.04.004.

Chaves, M.M., Pereira, J.S., Maroco, J., Rodrigues, M.L., Ricardo, C.P.P., Osório, M.L., Carvalho, I., Faria, T., Pinheiro, C., 2002. How plants cope with water stress in the field? Photosynthesis and growth. Ann. Bot. 89, 907–916. https://doi.org/10.1093/aob/mcf105.

Chen, D., Wang, S., Cao, B., Cao, D., Leng, G., Li, H., Yin, L., Shan, L., Deng, X., 2016. Genotypic variation in growth and physiological response to drought stress and re-watering reveals the critical role of recovery in drought adaptation in maize seedlings. Front. Plant Sci. 6, 1–15. https://doi.org/10.3389/fpls.2015.01241.

Chouchani, E.T., James, A.M., Fearnley, I.M., Lilley, K.S., Murphy, M.P., 2011. Proteomic approaches to the characterization of protein thiol modification. Curr. Opin. Chem. Biol. 15, 120–128. https://doi.org/10.1016/j.cbpa.2010.11.003.

Coupel-Ledru, A., Lebon, E., Christophe, A., Gallo, A., Gago, P., Pantin, F., Doligez, A., Simonneau, T., 2016. Reduced nighttime transpiration is a relevant breeding target for high water-use efficiency in grapevine. Proc. Natl. Acad. Sci. U.S.A. 113, 8963–8968. https://doi.org/10.1073/pnas.1600826113.

Da Costa, M., Huang, B., 2006. Changes in carbon partitioning and accumulation patterns during drought and recovery for colonial bentgrass, creeping bentgrass, and velvet bentgrass. J. Am. Soc. Hortic. Sci. 131, 484–490.

Denaxa, N.-K., Roussos, P.A., Damvakaris, T., Stournaras, V., 2012. Comparative effects of exogenous glycine betaine, kaolin clay particles and ambiol on photosynthesis, leaf sclerophylly indexes and heat load of olive cv. Chondrolia Chalkidikis under drought. Sci. Hortic. 137, 87–94. https://doi.org/10.1016/j.scienta.2012.01.012.

Dinis, L.-T., Bernardo, S., Conde, A., Pimentel, D., Ferreira, H., Félix, L., Gerós, H., Correia, C.M., Moutinho-Pereira, J., 2016. Kaolin exogenous application boosts antioxidant capacity and phenolic content in berries and leaves of grapevine under summer stress. J. Plant Physiol. 191, 45–53. https://doi.org/10.1016/j.jplph.2015.12.005.

Dinis, L.-T., Bernardo, S., Luzio, A., Pinto, G., Meijón, M., Pintó-Marijuan, M., Cotado, A., Correia, C., Moutinho-Pereira, J., 2018. Kaolin modulates ABA and IAA dynamics and physiology of grapevine under Mediterranean summer stress. J. Plant Physiol. 220, 181–192. https://doi.org/10.1016/j.jplph.2017.11.007.

Domonkos, I., Kis, M., Gombos, Z., Ughy, B., 2013. Carotenoids, versatile components of oxygenic photosynthesis. Prog. Lipid Res. 5, 539–561. https://doi.org/10.1016/j.plipres.2013.07.001.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77. https://doi.org/10.1016/0003-9861(59)90090-6.

Escalona, J.M., Fuentes, S., Tomás, M., Martorell, S., Flexas, J., Medrano, H., 2013. Responses of leaf night transpiration to drought stress in *Vitis vinifera* L. Agric. Water Manag. 118, 50–58. https://doi.org/10.1016/j.agwat.2012.11.018.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., Basra, S.M.A., 2009. Plant drought stress: efects, mechanisms and management. Agron. Sustain. Dev. 29, 185–212. https://doi.org/10.1051/agro:2008021.

Flexas, J., Medrano, H., 2002. Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. Ann. Bot. 89, 183–189. https://doi.org/10.1093/aob/mcf027.

Flexas, J., Ribas-Carbó, M., Bota, J., Galmés, J., Henkle, M., Martínez-Cañellas, S., Medrano, H., 2006. Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. New Phytol. 172, 73–82. https://doi.org/10.1111/j.1469-8137.2006.01794.x.

Genty, B., Briantais, J.M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta 990, 87–92. https://doi.org/10.1016/S0304-4165(89)80016-9.

Glenn, D.M., 2009. Particle film mechanisms of action that reduce the effect of environmental stress in ‘Empire’ apple. J. Am. Soc. Hortic. Sci. 134, 314–321.

Glenn, D.M., Puterka, G.J., 2005. Particle Films: a new technology for agriculture. In: Janick, J. (Ed.), Horticultural Reviews. John Wiley & Sons, Inc., pp. 1–44.

Gregoriou, K., Pontikis, K., Vemmos, S., 2007. Effects of reduced irradiance on leaf morphology, photosynthetic capacity, and fruit yield in olive (*Olea europaea* L.). Photosynthetica 45, 172–181. https://doi.org/10.1007/s11099-007-0029-x.

Gulen, H., Eris, A., 2004. Effect of heat stress on peroxidase activity and total protein content in strawberry plants. Plant Sci. 166, 739–744. https://doi.org/10.1016/j.plantsci.2003.11.014.

Gupta, S.D., Augé, R.M., Denchev, P.D., Conger, B.V., 1995. Growth, proline accumulation and water relations of NaCl-selected and non-selected callus lines of Dactylis glomerata L. Environ. Exp. Bot. 35, 83–92. https://doi.org/10.1016/0098-8472(94)E0011-R.

Hasanuzzaman, M., Kamrun Nahar, K., Alam, M.M., Roychowdhury, R., Fujita, M., 2013. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. Int. J. Mol. Sci. 14, 9643–9684. https://doi.org/10.3390/ijms14059643.

Howard, A.R., Donovan, L.A., 2007. Helianthus nighttime conductance and transpiration respond to soil water but not nutrient availability. Plant Physiol. 143, 145–155. https://doi.org/10.1104/pp.106.089383.

Hunt, R., 1978. Plant Growth Analysis. Edward Arnold, London, UK.

IPCC, 2013. In: Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.), Climate Change 2013: the Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, pp. 1535 New York.

IPMA, 2017. Instituto português do mar E da atmosfera. http://www.ipma.pt/pt/oclima/normais.clima/, Accessed date: 20 December 2017.

Irigoyen, J.J., Einerich, D.W., Sánchez-Díaz, M., 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativd*) plants. Physiol. Plantarum 84, 55–60. https://doi.org/10.1111/j.1399-3054.1992.tb08764.x.

Jifon, J.L., Syvertsen, J.P., 2003. Kaolin particle film applications can increase photosynthesis and water use efficiency of `Ruby Red' grapefruit leaves. J. Am. Soc. Hortic. Sci. 128, 107–112.

Junglee, S., Urban, L., Sallanon, H., Lopez-Lauri, F., 2014. Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. Am. J. Anal. Chem. 5, 730–736.

Klein, B.P., Perry, A.K., 1982. Ascorbic acid and vitamin a activity in selected vegetables from different geographical areas of the United States. J. Food Sci. 47, 941–945. https://doi.org/10.1111/j.1365-2621.1982.tb12750.x.

Kong, L., Wang, F., Si, J., Feng, B., Zhang, B., Li, S., Wang, Z., 2013. Increasing in ROS levels and callose deposition in peduncle vascular bundles of wheat (*Triticum aestivum* L.) grown under nitrogen deficiency. J. Plant Interact. 8, 109–116. https://doi.org/10.1080/17429145.2012.712723.

Krause, G.H., Weis, E., 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 313–349. https://doi.org/10.1146/annurev.pp.42.060191.001525.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In: Methods in Enzymology. Academic Press, pp. 350–382.

Lichtenthaler, H.K., Ač, A., Marek, M.V., Kalina, J., Urban, O., 2007. Differences in pigment composition, photosynthetic rates and chlorophyll fluorescence images of sun and shade leaves of four tree species. Plant Physiol. Biochem. 45, 577–588. https://doi.org/10.1016/j.plaphy.2007.04.006.

Lisar, S.Y.S., Motafakkerazad, R., Hossain, M.M., Rahman, I.M.M., 2012. Water stress in plants: causes, effects and responses, water stress. In: Rahman, I.M., Hasegawa, H. (Eds.), Water Stress. InTech, pp. 1–14.

Liu, J.-F., Arend, M., Yang, W.-J., Schaub, M., Ni, Y.-Y., Gessler, A., Jiang, Z.-P., Rigling, A., Li, M.-H., 2017. Sci. Rep. 7, 42462. https://doi.org/10.1038/srep42462.

Mattos, L.M., Moretti, C.L., 2015. Oxidative stress in plants under drought conditions and the role of different enzymes. Enzyme Eng. 5, 136. https://doi.org/10.4172/2329-6674.1000136.

Medrano, H., Tomás, M., Martorell, S., Flexas, J., Hernández, E., Rosselló, J., Pou, A., Escalona, J.-M., Bota, J., 2015. From leaf to whole-plant water use efficiency (WUE) in complex canopies: limitations of leaf WUE as a selection target. Crop J 3, 220–228. https://doi.org/10.1016/j.cj.2015.04.002.

Mena-Petite, A., Ortega-Lasuen, U., González-Moro, M.B., Lacuesta, M., Muñoz-Rueda, A., 2001. Storage duration and temperature effect on the functional integrity of container and bare-root *Pinus radiata* D. Don stock-types. Trees (Berl.) 15, 289–296. https://doi.org/10.1007/s004680100104.

Mullan, D., Pietragalla, J., 2011. Leaf relative water content. In: Pask, A., Pietragalla, J., Mullan, D., Reynolds, M. (Eds.), Physiological Breeding II: a Field Guide to Wheat Phenotyping CIMMYT International Maize and Wheat Improvement Center, Mexico, pp. 25–27.

Munné-Bosch, S., Peñuelas, J., 2003. Photo- and antioxidative protection, and a role for salicylic acid during drought and recovery in field-grown *Phillyrea angustifolia* plants. Planta 217, 758–766. https://doi.org/10.1007/s00425-003-1037-0.

Nanos, P.G., 2015. Leaf and fruit responses to kaolin particle film applied onto mature olive trees. J. Biol. Agric. Healthc. 5, 17–27.

Osaki, M., Shinano, T., Tadano, T., 1991. Redistribution of carbon and nitrogen compounds from the shoot to the harvesting organs during maturation in field crops. Soil Sci. Plant Nutr. 37, 117–128. https://doi.org/10.1080/00380768.1991.10415017.

Perez-Martin, A., Michelazzo, C., Torres-Ruiz, J.M., Flexas, J., Fernández, J.E., Sebastiani, L., Diaz-Espejo, A., 2014. Regulation of photosynthesis and stomatal and mesophyll conductance under water stress and recovery in olive trees: correlation with gene expression of carbonic anhydrase and aquaporins. J. Exp. Bot. 65, 3143–3156. https://doi.org/10.1093/jxb/eru160.

Petrov, V.D., Van Breusegem, F., 2012. Hydrogen peroxide—a central hub for information flow in plant cells. AoB Plants 2012 pls014. https://doi.org/10.1093/aobpla/pls014.

Rosati, A., Metcalf, S.G., Buchner, R.P., Fulton, A.E., Lampinen, B.D., 2006. Physiological effects of kaolin applications in well-irrigated and water-stressed walnut and almond trees. Ann. Bot. 98, 267–275. https://doi.org/10.1093/aob/mcl100.

Roussos, P.A., Denaxa, N.-K., Damvakaris, T., Stournaras, V., Argyrokastritis, I., 2010. Effect of alleviating products with different mode of action on physiology and yield of olive under drought. Sci. Hortic. 125, 700–711. https://doi.org/10.1016/j.scienta.2010.06.003.

Segura-Monroy, S., Uribe-Vallejo, A., Ramirez-Godoy, A., Restrepo-Diaz, H., 2015. Effect of kaolin application on growth, water use efficiency, and leaf epidermis characteristics of *Physallis peruviana* seedlings under two irrigation regimes. Agr. Sci. Tech. 17, 1585–1596.

Sesták, Z., Castky, J., Jarvis, P.G., 1971. Plant Photosynthetic Production. Manual of Methods. Dr. W. Junk Publishers, The Hague, Netherlands.

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 2012, 26. https://doi.org/10.1155/2012/217037.

Shellie, K.C., King, B.A., 2013. Kaolin-based foliar reflectant and water deficit influence malbec leaf and berry temperature, pigments, and photosynthesis. Am. J. Enol. Vitic. 64, 223–230. https://doi.org/10.5344/ajev.2012.12115.

Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–158.

Tognetti, R., Johnson, J., Michelozzi, M., 1995. The response of European beech (*Fagus sylvatica* L.) seedlings from two Italian populations to drought and recovery. Trees (Berl.) 9, 348–354. https://doi.org/10.1007/BF00202499.

Varone, L., Gratani, L., 2015. Leaf respiration responsiveness to induced water stress in Mediterranean species. Environ. Exp. Bot. 109, 141–150. https://doi.org/10.1016/j.envexpbot.2014.07.018.

von Caemmerer, S., Farquhar, G.D., 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153, 376–387. https://doi.org/10.1007/BF00384257.

Xu, B.J., Chang, S.K., 2007. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. J. Food Sci. 72, S159–S166. https://doi.org/10.1111/j.1750-3841.2006.00260.x.

Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2.